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(54) Title: A METHOD OF CLONING PROTEINS IN YEAST AND CELLULASE FROM HUMICOLA INSOLENS

(57) Abstract

Method of screening for a DNA sequence coding for a protein of interest, the method comprising a) cloning, in suitable vectors, a DNA library from an organism suspected of producing one or more proteins of interest; b) transforming suitable yeast host cells with said vectors; c) culturing the host cells under suitable conditions to express any protein of interest encoded by a clone in the DNA library, and d) screening for positive clones by determining any activity of a protein expressed in step (c). An enzyme which exhibits cellulase activity and has been isolated from DNA library of Humicola insolens. The enzyme has a cellulose binding domain and exhibits endocellulase activity in the presence of linear alkyl benzene sulfonate.

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A method of cloning proteins in yeast and cellulase from Humicola insolens.

FIELD OF INVENTION

The present invention relates to a method of screening for DNA sequences coding for proteins of interest, as well as to a process for producing such proteins of interest.

BACKGROUND OF THE INVENTION

The advent of recombinant DNA techniques has made it possible to select single protein components with interesting properties and produce them on a large scale. This represents an improvement over the previously employed production process using microorganisms isolated from nature and producing a mixture of proteins which would either be used as such or separated after the production step. However, the conventional cloning techniques have the drawback that each protein component has to be purified and characterized by its (partial) amino acid sequence before it is possible to prepare synthetic oligonucleotide probes for hybridization experiments. Since this is a rather time-consuming process, the cloning of novel proteins might be considerably expedited by using a screening method involving selecting clones expressing a desired protein activity.

Such a screening method has previously been devised for the cloning of prokaryotic gene products in _Bacillus_, cf. US 4,469,791; P. Cornelis et al., _Mol. Gen. Genet._ _186_, 1982, pp. 507-511; I. Palva, _Gene_ _19_, 1982, pp. 81-87; S.A. Ortlepp, _Gene_ _23_, 1983, pp. 267-276; H. Yamazaki et al., _J. Bacteriol._ _156_, 1983, pp. 327-337; N. Tsukagoshi et al., _Mol. Gen. Genet._ _193_, 1984, pp. 58-63; M. Sibakov and I. Palva, _Eur. J. Biochem._ _145_, 1984, pp. 567-572; and J.R. Mielenz, _Proc. Natl. Acad. Sci. USA_ _80_, 1983, pp. 5975-5979. A screening method based on expression cloning of eukaryotic genes in mammalian cells has been described, e.g. in D.P. Gearing et al., _The EMBO J._ _8_, 1989,

pp. 3667-3676; N. Harada et al., Proc. Natl. Acad. Sci. USA 87, 1990, pp. 857-861; and R. Fukunaga et al., Cell 61, 1990, pp. 341-350.

SUMMARY OF THE INVENTION

It has now been found possible to screen for yeast clones expressing protein activities of interest with a view to isolating DNA coding for single protein components.

Accordingly, the present invention relates to a method of screening for a DNA sequence coding for a protein of interest, the method comprising

(a) cloning, in suitable vectors, a DNA library from an organism suspected of producing one or more proteins of interest,

(b) transforming suitable yeast host cells with said vectors,

(c) culturing the host cells under suitable conditions to express any protein of interest encoded by a clone in the DNA library, and

(d) screening for positive clones by determining any activity of a protein expressed in step (c).

As indicated above, expression cloning of prokaryotic genes in Bacillus has previously been described. The prokaryotic systems devised for expression cloning, however, are not operable for the cloning of eukaryotic genes which are generally difficult to express in Bacillus. While expression cloning of eukaryotic genes in mammalian cells has been described, it is more advantageous to use yeast as a host organism as it is possible to obtain a much higher transformation frequency than with mammalian cells, and as yeast is far easier to cultivate. Furthermore, the yeast clones are stable whereas the mammalian

expression cloning system described in the references cited above is based on transient expression in COS cells. Unlike the mammalian system, the yeast system results in pure clones after the initial screening and, therefore, they need not be screened in pools and subpools as in the mammalian system. Apart from this conventional selection systems may be used to select yeast transformants.

According to the present invention, it has surprisingly been found that yeast cells appear to be able to express heterologous genes extracellularly by means of heterologous secretion signals in amounts which are sufficient for screening purposes. Although expression cloning of certain proteins in yeast has been described previously (G.L. McKnight and B.L. McConaughy, Proc. Nat. Acad. Sci. USA 80, 1983, pp. 4412-4416), it has not been generally useful as it is based on complementation of essential genes and therefore is dependent on yeast host strains which have been mutated to lack these essential genes. In the present screening method, no such requirement is necessary for the yeast host strain to be used in the method. Besides, the gene products of the previously described method are intracellular rather than extracellular as in the present method.

The advantage presented by the present screening method is primarily that it requires no prior knowledge of the structure of the protein of interest. This means that the rate at which novel genes may be isolated and, consequently, novel products be developed may be greatly increased. Furthermore, the method permits screening for multiple protein activities and may even result in the isolation of several different genes coding for the same type of proteins.

In another aspect, the present invention relates to a process for producing a protein of interest in a heterologous host cell, the process comprising transforming a suitable heterologous host cell with a DNA sequence coding for a protein

of interest, which DNA sequence has been isolated by the screening method of the invention, culturing the transformed cells under suitable conditions to express the protein, and recovering the expressed protein from the culture.

In a further aspect, the present invention relates to an enzyme which exhibits cellulase activity, and which has the following characteristics

(a) the DNA sequence encoding the enzyme has been isolated from a DNA library of _Humicola insolens_,

(b) said DNA sequence comprises at least one of the following partial sequences

(i) TGGCAGCAGT GTGGTGGCGT TGGCTTCTCG GGCTCTACGT CCTGTGTGTC CGGTTACACG TGCGTGTACT TGAACGACTG GTACAGCCAA TGC (SEQ ID#1)

(ii) CAGCGCAGCC GACGACGTTA CGGACAACAC AACAACGACC AGGGCAACAT CGACAACAAG GTCAGCCCCG GCTGCCACTT CAACCACTCCG G (SEQ ID#2)

(iii) CCAAGGCGAA GTTCAAGTGG TTGGCATCAA CCAGTCCTGC GCTGAGTTCG GCAAGGAGAG TATCCGGCTA TGGGCAAGCA CTTACTTCCT TCGCGACGTC GTCGATTCAA GCGCACATCA ATCGTGGCTT CA (SEQ ID#3)

(iv) CTGACGTGAA CGTGACCAAC AACAACTTGG CCGTAGCGAC CGAGAACAAG CTGTGTACCA GATGCATCA (SEQ ID#4)

(v) GGACGGTCCG GCACGAGCAC GGCCTGCGTC AGCACCCAGG TCGGCCTTCA GCGCGTCATT GGCGCGACCA ACTGGCTCAG GCAAAACGGC AAGGTTGGAC TGCTCGCGAC TTGCCGCGGC (SEQ ID#5)

(vi) GCCAAGTGGG TTTGCCAGCA GGCCATTGAG GGCATGCTGA ACCACCTCCA GGAGAATAGC GATGTCTGGA CAGGTGCGCT CTGGTGGGCG GGAGGCCCGT GGTGGGGTTG ACTATATCTA (SEQ ID#6)

(c) the enzyme comprises a cellulose-binding domain, and

(d) the enzyme exhibits endocellulase activity in the presence of linear alkyl benzene sulfonate.

The enzyme of the invention may be isolated by the method of the invention.

In the present context, the term "cellulose-binding domain" is intended to indicate an amino acid sequence capable of effecting binding of the enzyme to a cellulosic substrate. Cellulose-binding domains have been found to be important for the endoglucanase activity of cellulytic enzymes on substrates (cf. the discussion in PCT/DK91/00124). The term "endocellulase activity" refers to the ability of the enzyme to degrade cellulose to glucose, cellobiose, triose and other cello-oligosaccharides, as determined by the formation of clearing zones in a carboxymethyl cellulose (CMC) gel under the conditions specified below. Unlike the endocellulase described in PCT/DK91/00123), the enzyme of the present invention shows substantially unchanged stability in the presence of linear alkyl benzene sulfonates. This is an important advantage as linear alkyl benzene sulfonates are commonly used in detergent compositions.

DETAILED DISCLOSURE OF THE INVENTION

According to the invention, the DNA library is preferably a cDNA library prepared from the mRNA of an organism suspected of producing one or more proteins of interest. Although it may also be possible to screen genomic libraries in this manner, at least some potential yeast hosts may not be able to splice

eukaryotic genomic DNA correctly, and therefore a positive result of the screening may more often be obtained by using cDNA instead.

To ensure a more accurate result, it may be an advantage to subject positive clones isolated in step (d) of the present method to rescreening, reisolation and recloning.

The organism suspected of producing one or more proteins of interest is typically a eukaryotic organism, in particular a fungus since fungi are known to produce a large number of different proteins which makes the traditional process of isolating a gene coding for a particular protein product by initially purifying each protein separately particularly cumbersome. This makes it particularly advantageous to screen fungal DNA libraries by the method of the invention because a large number of different protein activities (and DNAs coding for them) may be identified within a relatively short time-span using the same library. In this respect, screening of yeast colonies for different protein activities is far more efficient than screening of filamentous fungi as a large number (i.e. about 500-1000) of yeast colonies may be grown on each plate, compared to 10-50 filamentous fungi/plate.

One type of industrially useful proteins currently obtained from fungi is enzymes. Thus, yeast clones may be screened by the method of the invention for expression of one or more enzyme activities by means of appropriate assays. Examples of enzymes which may be identified by this method are carbohydrases, e.g. cellulytic enzymes such as endocellulases, cellobiohydrolases, β-glucanases or β-glucosidases, hemicellulases or pectinolytic enzymes such as galactanases, galactosidases, mannanases, xylanases, pectinases, xylosidases, arabanases, rhamnogalacturonases or amylases; esterases, e.g. lipolytic enzymes such as lipases; proteases; oxidoreductases, e.g. peroxidases, oxidases or laccases; or isomerases, e.g. glucose isomerase.

A wide range of indicator systems for the different types of enzymes may be used for the screening of yeast colonies on agar plates. For instance, endocellulases may be identified by clearing zones in carboxymethyl cellulose after staining with Congo Red; similar methods may be used to detect glucanases, xylanases and galactanases. Endoarabanases may be identified by blue zones obtained after dissolution of azurine-crosslinked araban. This principle is general and may be used to detect, e.g., mannanases, xylanases and cellulases. Pectinases (polygalacturonases and pectin lyases) may be identified by clearing zones in pectin after precipitation with quaternary ammonium ions. Amylases may be identified by clearing zones in starch after visualisation with iodine. α-galactosidases may be detected by the release of p-nitrophenol (yellow) from p-nitrophenol-α-galactopyranoside or by coupling released naphthole or naphthole derivatives from, e.g., 1-naphthole-α-galactopyranoside to azo dyes; similar methods may be used to detect β-galactosidases, α- and β-glycosidases, β-xylosidase and β-mannosidase. Numerous methods are available for the detection of proteases, e.g. clearing zones in casein after precipitation with trichloroacetic acid. Peroxidases and oxidases may be detected by the reaction of 4-aminoantipyrine with ESBT (N-ethyl-N-sulfobutyl-m-toluidine) in the presence of hydrogen peroxide (generating a purple colour). Lipases may be detected by the formation of clearing zones in tributyrine emulsions.

The yeast strain selected to be the host cell for the DNA library may be any yeast strain conventionally used for the cloning of heterologous DNA sequences. Thus, the yeast strain may suitably be selected from Saccharomyces sp., such as Saccharomyces cerevisiae, Saccharomyces kluyveri, Saccharomyces uvarum or Schizosaccharomyces pombe, Hansenula sp. Pichia sp., Yarrowia sp. such as Yarrowia lipolytica, or Kluyveromyces sp. such as Kluyveromyces lactis.

The vector used for cloning the DNA library may be any vector which may conveniently be subjected to recombinant DNA procedures. In each vector, the DNA sequence derived from the library should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the yeast cell. Examples of suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255, 1980, pp. 12073-12080; Alber and Kawasaki, J. Mol. Appl. Gen. 1, 1982, pp. 419-434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4, 599, 311) or ADH2-4c (Russell et al., Nature 304, 1983, pp. 652-654) promoters.

Each DNA library sequence may also be operably connected to a suitable terminator, such as the TPI1 (Alber and Kawasaki, op. cit.) or ADH3 (McKnight et al., op. cit.) or yeast MFα terminators.

The vector may further comprise a DNA sequence enabling the vector to replicate in yeast cell. An example of such a sequence is the yeast plasmid 2μ replication genes REP 1-3 and origin of replication. If the vector is a yeast/E. coli shuttle vector, it will also include an origin of replication region which is functional in E. coli. The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell such as URA3, or one which confers resistance to a drug, e.g. ampicillin, kanamycin, chloramphenicol, tetracyclin, etc., or the Schizosaccharomyces pombe TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130).

The procedures used to ligate the DNA library sequences, the promoter and the terminator, respectively, and to introduce them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art

(cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1989). The transformation of yeast cells may for instance be effected by protoplast formation followed by transformation or by the LiAc method in a manner known *per se*.

In the process of the invention of producing a protein of interest after the DNA coding for the protein has been isolated by the screening method described above, the heterologous host cell transformed with the isolated DNA sequence may be a strain of a filamentous fungus, e.g. fungi belonging to the groups Phycomycetes, Zygomycetes, Ascomycetes, Basidiomycetes or Fungi Imperfecti, including Hyphomycetes such as the genera *Aspergillus*, *Trichoderma*, *Penicillium*, *Fusarium* or *Humicola*.

The filamentous fungus host organism may conveniently be one which has previously been used as a host for producing recombinant proteins, e.g. a strain of *Aspergillus* sp., such as *A. niger*, *A. nidulans* or *A. oryzae*. The use of *A. oryzae* in the production of recombinant proteins is extensively described in, e.g. EP 238 023.

In particular when the host organism is *A. oryzae*, a preferred promoter for use in the process of the present invention is the *A. oryzae* TAKA amylase promoter as it exhibits a strong transcriptional activity in *A. oryzae*. The sequence of the TAKA amylase promoter appears from EP 238 023.

Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The techniques used to transform a fungal host cell may suitably be as described in EP 238 023.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing *Aspergillus* cells. The mature protein secreted from the host cells may

conveniently be recovered from the culture medium by well-known procedures including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

A preferred endocellulase enzyme according to the invention is an enzyme, a crude extract (15 μl) of which diluted with one volume of 0.15% linear alkyl benzene sulfonate and added to a 2% agarose gel containing 1% carboxymethyl cellulose in 50 mM sodium phosphate buffer, pH 7, mixed with one volume of 0.15% linear alkyl sulfonate forms a clearing zone in said agarose gel after 18 hours of incubation, which clearing zone is equal to (less 3 mm) the clearing zone formed in a similar carboxymethyl cellulose gel not containing any linear alkyl benzene sulfonate, provided that the concentration of enzyme in the extract is such that a clearing zone of at least 10 mm is formed in a carboxymethyl cellulose gel (with no linear alkyl benzene sulfonate) under the conditions specified above.

The DNA sequence coding for the enzyme may for instance be isolated by screening a cDNA library of Humicola insolens, e.g strain DSM 1800, deposited on 1 October 1981 at the Deutsche Sammlung von Mikroorganismen in accordance with the provisions of the Budapest Treaty and selecting for clones expressing the appropriate enzyme activity (i.e. endocellulase activity as defined above). The appropriate DNA sequence may then be isolated from the clone by standard procedures, e.g. as described in Example 1.

In a further aspect, the invention relates to a detergent additive comprising the enzyme of the invention. The detergent additive may suitably be in the form of a non-dusting granulate, stabilized liquid or protected enzyme. Non-dusting granulates may be produced e.g. according to US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be

coated by methods known in the art. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238 216.

It will be understood that the detergent additive may further include one or more other enzymes, such as a protease, lipase, peroxidase or amylase, conventionally included in detergent additives.

In a still further aspect, the present invention relates to a detergent composition comprising the enzyme of the invention. The detergent composition of the invention may be in any convenient form, e.g. as powder, granules or liquid. A liquid detergent may be aqueous, typically containing up to 90% water and 0-20% organic solvent.

The detergent composition comprises a surfactant which may be anionic, non-ionic, cationic, amphoteric or a mixture of these types. The detergent will usually contain 0-50% anionic surfactant such as linear alkyl benzene sulphonate (LAS), alpha-olefin sulphonate (AOS), alkyl sulphate (AS), alcohol ethoxy sulphate (AES) or soap. It may also contain 0-40% non-ionic surfactant such as nonyl phenol ethoxylate or alcohol ethoxylate. Furthermore, it may contain a polyhydroxy fatty acid amide surfactant (e.g. as described in WO 92/06154).

The detergent composition may additionally comprise one or more other enzymes, such as an amylase, lipase, peroxidase, oxidase or protease.

The pH (measured in aqueous detergent solution) will usually be neutral or alkaline, e.g. 7-11. The detergent may contain 1-40% of a detergent builder such as zeolite, phosphate, phosphonate, citrate, NTA, EDTA or DTPA, alkenyl succinic

anhydride, or silicate, or it may be unbuilt (i.e. essentially free from a detergent builder). It may also contain other conventional detergent ingredients, e.g. fabric conditioners, foam boosters, bleaching agents, e.g. perborate, percarbonate, tetraacetyl ethylene diamine (TAED), or nonanoyloxybenzene sulfonate (NOBS), anti-corrosion agents, soil-suspending agents, sequestering agents, anti-soil redeposition agents, stabilizing agents for the enzyme(s), foam depressors, dyes, bactericides, optical brighteners or perfumes.

Particular forms of detergent composition within the scope of the invention include:

a) A detergent composition formulated as a detergent powder containing phosphate builder, anionic surfactant, nonionic surfactant, silicate, alkali to adjust to desired pH in use, and neutral inorganic salt.

b) A detergent composition formulated as a detergent powder containing zeolite builder, anionic surfactant, nonionic surfactant, acrylic or equivalent polymer, silicate, alkali to adjust to desired pH in use, and neutral inorganic salt.

c) A detergent composition formulated as an aqueous detergent liquid comprising anionic surfactant, nonionic surfactant, humectant, organic acid, caustic alkali, with a pH in use adjusted to a value between 7 and 10.5.

d) A detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant consisting essentially of linear alkoxylated primary alcohol, phosphate builder, caustic alkali, with a pH in use adjusted to a value between about 7 and 10.5.

e) A detergent composition formulated as a detergent powder in the form of a granulate having a bulk density of at least 600 g/l, containing anionic surfactant and nonionic surfactant,

low or substantially zero neutral inorganic salt, phosphate builder, and sodium silicate.

f) A detergent composition formulated as a detergent powder in the form of a granulate having a bulk density of at least 600 g/l, containing anionic surfactant and nonionic surfactant, low or substantially zero neutral inorganic salt, zeolite builder, and sodium silicate.

g) A detergent composition formulated as a detergent powder containing anionic surfactant, nonionic surfactant, acrylic polymer, fatty acid soap, sodium carbonate, sodium sulphate, clay particles, and sodium silicate.

h) A liquid compact detergent comprising 5-65% by weight of surfactant, 0-50% by weight of builder and 0-30% by weight of electrolyte.

Apart from these ingredients, the detergent compositions a)-h) include the cellulase of the invention and optionally one or more other enzymes, as indicated above.

The softening, soil removal and colour clarification effects obtainable by means of the enzyme of the invention generally require a concentration of the enzyme in the washing solution of 0.0001 - 100, preferably 0.0005 - 60, and most preferably 0.01 - 20 mg of enzyme protein per liter. The detergent composition of the invention is typically employed in concentrations of 0.5 - 20 g/l in the washing solution. In general, it is most convenient to add the detergent additive in amounts of 0.1 - 5% w/w or, preferably, in amounts of 0.2 - 2% of the detergent composition.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a map of plasmid pYHD17, wherein "TPI promoter" indicates the <u>S</u>. <u>cerevisiae</u> triose phosphate isomerase

promoter, "Terminator" indicates the _S._ _cerevisiae_ triose phosphate isomerase terminator, "Amp" indicates the gene mediating ampicillin resistance, "2μ ori" indicates the yeast plasmid 2μ origin of replication, and "URA3" indicates a gene encoding a selection marker complementing a uracil deficiency in the host strain; and

Fig. 2 is a map of plasmid pHD414, wherein "AMG Terminator" indicates the _A._ _niger_ glucoamylase terminator, and "TAKA Promoter" indicates the _A._ _oryzae_ TAKA amylase promoter;

The present invention is further illustrated in the following examples which are not in any way intended to limit the scope of the invention as claimed.

EXAMPLES

Materials and Methods

Donor organism: mRNA was isolated from the following organisms: _H. insolens_, DSM 1800, grown in a cellulose-rich fermentation medium with agitation to ensure sufficient aeration.

Construction of an expression plasmid: The commercially available plasmid pYES II (Invitrogen) was cut with SpeI, filled in with Klenow DNA polymerase + dNTP and cut with ClaI. The DNA was size fractionated on an agarose gel, and a fragment of about 2000 bp was purified by electroelution. The same plasmid was cut with ClaI/PvuII, and a fragment of about 3400 bp was purified by electroelution. The two fragments were ligated to a blunt-ended SphI/EcoRI fragment containing the yeast TPI promoter. This fragment was isolated from a plasmid in which the TPI promoter from _S. cerevisiae_ (cf. T. Albers and G. Kawasaki, _J. Mol. Appl. Genet._ _1_, 1982, pp. 419-434) was slightly modified: an internal SphI site was removed by deleting the four bp constituting the core of this site. Furthermore, redundant sequences upstream of the promoter were

removed by Bal1 exonuclease treatment followed by addition of a SphI linker. Finally, an EcoRI linker was added at position -10. After these modifications, the promoter is included in a SphI-EcoRI fragment. Its effeciency compared to the original promoter appears to be unaffected by the modifications. The resulting plasmid pYHD17 is shown in Fig. 1.

Isolation of mRNA: Total RNA was isolated from approximately 7 g of mycelium. The mycelium was frozen in liquid nitrogen and ground in a mortar with 1 g of quartz sand to a consistency of flour. The RNA was extracted with guanidinium thiocyanate and centrifuged through CsCl essentially as described in Sambrook et al., 1989, op. cit.. Poly A RNA was isolated from total RNA by chromatrography on oligo dT cellulose.

cDNA synthesis: cDNA synthesis was carried out by means of a cDNA synthesis kit from Invitrogen according to the manufacturer's specifications. The DNA was adapted to the expression vectors by addition of a BstxI linker (Invitrogen) and size fractionated on an agarose gel. Only DNA larger than 5-600 bp was used in the library construction. The adapted cDNA was ligated into an appropriate vector cut with BstxI. Following test ligations (in order to determine the size of the library) the library was plated onto 50 agar plates. To each plate containing from approximately 500 to 5000 individual clones (dependent on the library size) was added 3 ml medium. The bacteria were scraped off, 1 ml glycerol was added, and stored at -80°C as 50 pools. The remaining 2 ml were used for DNA isolation. If the amount of DNA was insufficient to give the required number of yeast transformants (see below), large scale DNA was prepared from 500ml medium (TB) inoculated with 50 μl -80°C bacterial stock propagated over night.

Construction of Yeast Libraries: DNA from one or more pools was transformed into yeast as described below. To ensure that all the bacterial clones were tested in yeast a number of yeast

transformants 5 x larger than the number of bacteria clones in the original pools was set as a limit.

Transformation of yeast: The yeast strain used was yNG231. (MAT alpha, leu2, ura3-52, his4-539, pep4-delta 1, cir+). One colony was grown at 30°C overnight in 10 ml YPD (this culture can be stored for several days at 5°C).

10, 30, and 60 µl of this culture were added to 3 shaker flasks containing 100 ml YPD, and incubated with shaking overnight at 30°C. The culture with an OD_{600} closest to 0.3-0.4 was selected. The cells were harvested in 50 ml tubes in a Beckman centrifuge (speed 6, 10 minutes), the cells were resuspended in 2 x 5 ml H_2O, centrifuged as described above, resuspended in 5 ml buffer containing 0.1 M LiAc, 10 mM Tris-Cl, 1 mM EDTA, pH 7.5, and centrifuged again. The cells were resuspended in 500 µl of the above buffer and incubated for 60 minutes at 30°C. 250 µg carrier DNA (sterile salmon-sperm DNA 10 mg/ml) was added and aliquots of 100 µl were prepared. The DNA to be transformed (approx. 5 µg) was added to the 100 µl aliquot, mixed gently, and incubated for 30 minutes at 30°C. 700 µl 40% PEG 4000, 0.1 M LiAc, 10 mM Tris-Cl, 1 mM EDTA, pH 7.5 was added, and incubation was continued for 60 minutes at 30°C. The transformation mixture was subjected to heat shock for 5 minutes at 42°C, spun briefly in a micro centrifuge, resuspended in 100-200 µl H_2O, and plated on SC plates without uracil, followed by incubation for three days at 30°C.

Preparation of carrier DNA: 100 mg salmon-sperm DNA was weighed out and dissolved overnight in 10 ml 10 mM Tris-Cl, 1 mM EDTA, pH 7,5 (TE). The solution was then sonicated in a plastic container in ice water until it was no longer viscous. The solution was then phenole extracted and EtOH precipitated, and the pellet was washed and resuspended in 5 ml TE. The suspension was EtOH precipitated, and the pellet was washed and resuspend in 5 ml TE. The OD_{260} was measured, and the suspension was diluted with TE to 10 mg/ml.

Media:

YPD: 10 g yeast extract, 20 g peptone, H_2O to 810 ml. Autoclaved, 90 ml 20% glucose (sterile filtered) added.

10 x Basal salt: 66.8 g yeast nitrogen base, 100 g succinic acid, 60 g NaOH, H_2O ad 1000 ml, sterile filtered.

SC-URA: 90 ml 10 x Basal salt, 22.5 ml 20 % casamino acids, 9 ml 1% tryptophane, H_2O ad 806 ml, autoclaved, 3.6 ml 5% threonine and 90 ml 20% glucose added.

SC-H agar: 7.5 g/l yeast nitrogen base without amino acids, 11.3 g/l succinic acid, 6.8 g/l NaOH, 5.6 g/l casamino acids without vitamins, 0.1 g/l tryptophan and 20 g/l agar (Bacto). Autoclaved for 20 min. at 121°C. After autoclaving, 55 ml of a 22% galactose solution and 1.8 ml of a 5% threonine solution were added per 450 ml agar.

SC-H broth: 7.5 g/l yeast nitrogen base without amino acids, 11.3 g/l succinic acid, 6.8 g/l NaOH, 5.6 g/l casamino acids without vitamins, 0.1 g/l tryptophan. Autoclaved for 20 min. at 121°C. After autoclaving, 10 ml of a 30% galactose solution, 5 ml of a 30% glucose solution and 0.4 ml of a 5% threonine solution were added per 100 ml medium.

YNB-1 agar: 3.3 g/l KH_2PO_4, 16.7 g/l agar, pH adjusted to 7. Autoclaved for 20 min. at 121°C. After autoclaving, 25 ml of a 13.6% yeast nitrogen base without amino acids, 25 ml of a 40% glucose solution, 1.5 ml of a 1% L-leucine solution and 1.5 ml of a 1% histidine solution were added per 450 ml agar.

YNB-1 broth: Composition as YNB-1 agar, but without the agar.

CMC overlayer gel: 1% agarose, 1% carboxymethyl cellulose in Tris-malate buffer, pH 7. The gel was boiled and then cooled to 55°C before the overlayer was poured onto agar plates.

Oat spelt xylan overlayer gel: 1% agarose, 1% oat spelt xylan (Sigma Chemical Company) in Tris-malate buffer, pH 7. The gel was boiled and then cooled to 55°C before the overlayer is poured onto agar plates.

Construction of an _Aspergillus_ expression vector: The vector pHD414 is a derivative of the plasmid p775 (described in EP 238 023). In contrast to this plasmid, pHD 414 has a string of unique restriction sites between the promoter and the terminator. The plasmid was constructed by removal of an approximately 200 bp long fragment (containing undesirable RE sites) at the 3'end of the terminator, and subsequent removal of an approximately 250 bp long fragment at the 5'end of the promoter, also containing undesirable sites. The 200 bp region was removed by cleavage with NarI (positioned in the pUC vector) and XbaI (just 3' to the terminator), subsequent filling in the generated ends with Klenow DNA polymerase +dNTP, purification of the vector fragment on gel and religation of the vector fragment. This plasmid was called pHD413. pHD413 was cut with StuI (positioned in the 5'end of the promoter) and PvuII (in the pUC vector), fractionated on gel and religated. The plasmid pHD 414 is shown in Fig. 2.

<u>Example 1</u>

A library from _H. insolens_ consisting of approx. 300,000 individual clones in 50 pools was constructed.

DNA was isolated from 20 individual clones from the library and subjected to analysis for cDNA insertion. The insertion frequency was found to be >90 % and the average insert size was approximately 1400bp.

DNA was isolated from 10 pools from the <u>Humicola</u> library (2ml from the original plate). An aliquot was digested with restriction enzymes in order to excise the cDNA insert and analyzed by Southern blot using a 43kD cellulase cDNA probe

(the 43 kD enzyme is disclosed in PCT/DK91/00123)and a CBH 2 cDNA probe (the enzyme is disclosed in PCT/DK91/00124). Several bands were found to hybridize with the 43kD cellulase probe after a low stringency wash (2x SSC 65°C) in the 10 pools from the Humicola library. At higher stringency (0.1 x SSC, 75°C) one band corresponding to the expected size for 43kD cellulase was detected in 5 out of 10 pools. Similar results were obtained with the CBH 2 probe. Here 10 out of 10 pools were found to have a band corresponding to the expected size for CBH 2. In addition, 4 pools contained bands with a higher molecular weight. These bands were seen even under stringent conditions, demonstrating that the library is of an appropriately high quality.

DNA from the Humicola library, pools 1-10, was transformed into yeast, and plates containing 20-25,000 colonies were obtained from each pool. The colonies were scraped off and stored in glycerol at -80°C.

Yeast cells from the library were spread onto YNB agar to a total of about 400,000 colonies. The number of colonies per plate varied from 50 to 500. After 4 or 5 days of growth, the agar plates were replica plated onto two sets of SC-H agar plates. These plates were then incubated for 2-4 days at 30°C before the two sets of agar plates were overlayered with a CMC indicator gel for detection of cellulase activity and oat spelt xylan indicator gel for the detection of xylanase and cellulase. After incubation overnight at 40°C, enzyme reactions were visualised with Congo Red. 10-15 ml of a 0.1% solution of Congo Red was poured onto the overlayer and removed after 10-20 min. The plates were then washed once or twice by pouring 10-15 ml of 2M NaCl onto the plates. The NaCl solution was removed after 15-25 min. Cellulase-positive colonies were identified on the plates with the CMC overlayer as colonies with colourless or pale red clearing zones on a red background. Xylanase-positive colonies identified on the plates with oat spelt xylan overlayers as colourless or pale red clearing zones

on a red background. Cellulase-positive colonies were also identified on plates with oat spelt xylan overlayers as pale red or blue clearing zones on a red background.

Cells from enzyme-positive colonies were spread for single colony isolation on agar, and an enzyme-producing single colony was selected for each of the cellulase- or xylanase-producing colonies identified.

Each of the 133 cellulase-producing colonies and 147 of the xylanase-producing colonies were isolated. Some of these colonies were inoculated into 20 ml YNB-1 broth in a 50 ml glass test tube. The tube was shaken for 2 days at 30°C. The cells were harvested by centrifugation for 10 min. at 3000 rpm.

The cells were resuspended in 1 ml 0.9 M sorbitol, 0.1 M EDTA, pH 7.5. The pellet was transferred to an Eppendorf tube, and spun for 30 seconds at full speed. The cells were resuspended in 0.4 ml 0.9 M sorbitol, 0.1 M EDTA, 14 mM β-mercaptoethanol. 100 μl 2 mg/ml Zymolase was added, and the suspension was incubated at 37°C for 30 minutes and spun for 30 seconds. The pellet (spheroplasts) was resuspended in 0.4 ml TE. 90 μl of (1.5 ml 0.5 M EDTA pH 8.0, 0.6 ml 2 M Tris-Cl pH 8.0, 0.6 ml 10% SDS) was added, and the suspension was incubated at 65°C for 30 minutes. 80 μl 5 M KOAc was added, and the suspension was incubated on ice for at least 60 minutes and spun for 15 minutes at full speed. The supernatant was transferred to a fresh tube which was filled with EtOH (room temp.) followed by thorough but gentle mixing and spinning for 30 seconds. The pellet was washed with cold 70% ETOH, spun for 30 seconds and dried at room temperature. The pellet was resuspended in 50 μl TE and spun for 15 minutes. The supernatant was transferred to a fresh tube. 2.5 μl 10 mg/ml RNase was added, followed by incubation at 37°C for 30 minutes and addition of 500 μl isopropanol with gentle mixing. The mixture was spun for 30 seconds, and the supernatant was removed. The pellet was rinsed with cold 96% EtOH and dried at room temperature. The DNA was

dissolved in 50 μl water to a final concentration of approximately 100 $\mu l/ml$.

The DNA was transformed into E.coli. by standard procedures. Two E. coli colonies were isolated from each of the transformations and analysed with the restriction enzymes HindIII and XbaI which excised the DNA insert. DNA from one of these clones was retransformed into S. cerevisiae strain JG169 (MATα; ura 3-52; leu 2-3, 112; his 3-D200; pep 4-113; prc1::HIS3; prb1:: LEU2) and rescreened for enzyme activity.

The DNA sequences of several of the positive clones were partially determined. The partial DNA sequences are shown in Sequence Listings SEQ ID#7-15. Based on the DNA sequence, the clones were classified as follows:

Endocellulases:

CMC 1:	C3, 26, 27, XY33, XY46	250 amino acids (SEQ ID#7)
CMC 4:	C46, 47, 50, 51, 54, 101, 102, 103, 104	~1400 bp (the enzyme of the invention) (SEQ ID#8)
CMC 5:	XY49	~1050 bp (SEQ ID#9)
CMC 6:	C49	~1000 bp (SEQ ID#10)
CMC 38K:	C13	(SEQ ID#11)
CMC EG1:	C6, 11, 15, 16, 17, 21, 22, 23, 25, XY34, 41, 145	(SEQ ID#12)

Xylanases:

XYL 1:	XY30, 31, 40, 42, 101, 102, 110, 117, 119, 123, 125, 136, XY56, 60, 137	22 kD (SEQ ID#13)
XYL 2:	XY103, 104, 107, 108, 109, 113, 114, 118, 120, 121, 124, 126, 128, 130, 134, 142, 143	(SEQ ID#14)
XYL 3:	XY115, 116, 132, 146	(SEQ ID#15)

In order to express the genes in _Aspergillus_ the cDNA insert is isolated from one or more representatives of each family and cloned into the vector pHD414 which is transformed into _A. oryzae_ or _A. niger_ according to the general procedure described below.

Transformation of Aspergillus oryzae or Aspergillus niger (general procedure)

100 ml of YPD (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) is inoculated with spores of _A. oryzae_ or _A. niger_ and incubated with shaking at 37°C for about 2 days. The mycelium is harvested by filtration through miracloth and washed with 200 ml of 0.6 M $MgSO_4$. The mycelium is suspended in 15 ml of 1.2 M $MgSO_4$. 10 mM NaH_2PO_4, pH = 5.8. The suspension is cooled on ice and 1 ml of buffer containing 120 mg of Novozym® 234, batch 1687 is added. After 5 minutes 1 ml of 12 mg/ml BSA (Sigma type H25) is added and incubation with gentle agitation continued for 1.5-2.5 hours at 37°C until a large number of protoplasts is visible in a sample inspected under the microscope.

The suspension is filtered through miracloth, the filtrate transferred to a sterile tube and overlayered with 5 ml of 0.6 M sorbitol, 100 mM Tris-HCl, pH = 7.0. Centrifugation is performed for 15 minutes at 100 g and the protoplasts are collected from the top of the $MgSO_4$ cushion. 2 volumes of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5. 10 mM $CaCl_2$) are added to the protoplast suspension and the mixture is centrifugated for 5 minutes at 1000 g. The protoplast pellet is resuspended in 3 ml of STC and repelleted. This is repeated. Finally the protoplasts are resuspended in 0.2-1 ml of STC.

100 µl of protoplast suspension is mixed with 5-25 µg of the appropriate DNA in 10 µl of STC. Protoplasts are mixed with p3SR2 (an _A. nidulans_ amdS gene carrying plasmid). The mixture

is left at room temperature for 25 minutes. 0.2 ml of 60% PEG 4000 (BDH 29576). 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH = 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution is added and carefully mixed. The mixture is left at room temperature for 25 minutes, spun at 2500 g for 15 minutes and the pellet is resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are spread on the appropriate plates. Protoplasts are spread on minimal plates (Cove Biochem.Biophys.Acta <u>113</u> (1966) 51-56) containing 1.0 M sucrose, pH = 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked and spread for single colonies. This procedure is repeated and spores of a single colony after the second reisolation is stored as a defined transformant.

Example 2

Cellulase type 4 clones C46 and C51 and a 43 kD cellulase control clone (obtained by transforming yeast strain JG169 with pYHD17 carrying a DNA sequence coding for the 43 kD cellulase [isolated as described in PCT/DK91/000123]) were inoculated in 100 ml test tubes with 15 ml YNB-1 broth. The tubes were agitated at 30°C for 2 days. 5 ml of broth from each tube were then used as seed material for shake flasks containing 100 ml SC-H broth. The shake flasks were agitated for 4 days at 30°C. The cells from 20 ml of broth were collected by centrifugation and mixed with 1-2 ml 0.1 M sodium phosphate buffer, pH 7, and 3.3 g of glass beads (420-500 μm in diameter) in 10 ml glass test tubes. The crude cell extracts were collected after about 8 minutes of agitation by means of a IKA vibrax VXR (available from IKA Labortechnik).

The cellulase activity of the crude cell extracts from the yeast clones C46, C51 and 43 kD were measured under different conditions by the size of the clearing zones formed in CMC containing gels.

CMC gel: CMC overlayer gel as described above.

CMC LAS gel: 2% agarose, 1% CMC in 50 mM sodium phosphate buffer, pH 7, boiled and mixed with one volume of 0.12% LAS.

The cellulase activity was measured by adding 15 µl crude cell extract to 4 mm (diameter) holes in the gel. The crude cell extracts were diluted with one volume of 0.12% LAS before addition to the CMC LAS gel and with one volume of water before addition to the CMC gel. The clearing zones were then visualised after 18 hours of incubation at 40°C by staining with Congo Red as described above.

The results are shown in the following table.

Clone	C46	C51	43 kD
CMC	14	14	17
CMC LAS	15	13	0

Activities are shown as mm clearing zones.

It appears from the table that the enzyme produced by C46/C51 is LAS resistant.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45 4444 8888
(H) TELEFAX: +45 4449 3256
(I) TELEX: 37304

(ii) TITLE OF INVENTION: A Method of Cloning Proteins in Yeast

(iii) NUMBER OF SEQUENCES: 15

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 93 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Humicola insolens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TGGCAGCAGT GTGGTGGCGT TGGCTTCTCG GGCTCTACGT CCTGTGTGTC CGGTTACACG 60

TGCGTGTACT TGAACGACTG GTACAGCCAA TGC 93

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 92 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Humicola insolens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CAGCGCAGCC GACGACGTTA CGGACAACAC AACAACGACC AGGGCAACAT CGACAACAAG 60

GTCAGCCCCG GCTGCCACTT CAACCACTCC GG 92

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 132 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Humicola insolens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCAAGGCGAA GTTCAAGTGG TTGGCATCAA CCAGTCCTGC GCTGAGTTCG GCAAGGAGAG 60

TATCCGGCTA TGGCAAGCA CTTACTTCCT TCGCGACGTC GTCGATTCAA GCGCACATCA 120

ATCGTGGCTT CA 132

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Humicola insolens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CTGACGTGAA CGTGACCAAC AACAACTTGG CCGTAGCGAC CGAGAACAAG CTGTGTACCA 60

GATGCATCA 69

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Humicola insolens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GGACGGTCCG GCACGAGCAC GGCCTGCGTC AGCACCCAGG TCGGCCTTCA GCGCGTCATT 60

GGCGCGACCA ACTGGCTCAG GCAAAACGGC AAGGTTGGAC TGCTCGCGAC TTGCCGCGGC 120

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Humicola insolens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GCCAAGTGGG TTTGCCAGCA GGCCATTGAG GGCATGCTGA ACCACCTCCA GGAGAATAGC 60

GATGTCTGGA CAGGTGCGCT CTGGTGGGCG GGAGGCCCGT GGTGGGGTTG ACTATATCTA 120

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1027 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Humicola insolens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
CATCGCCTTA TACCACCAGC TCTACTGCAG ACCTTGTCCA ATTTCTCGGA TCACCGCCAT    60
GCTCAAGTCT GCTCTCCTCC TCGGGCCCGC GGCCGTTTCG GTTCAGTCGG CTTCGATCCC   120
GACCATCCCG GCAAACCTTG AGCCTCGCCA GATTCGCTCG CTCTGTGAGC TGTACGGCTA   180
CTGGTCCGGC AATGGCTATG AGCTGTTGAA CAACCTCTGG GGCAAGGACA CCGCCACATC   240
CGGCTGGCAA TGCACCTACC TCGACGGCAC CAACAACGGT GGCATTCAGT GGAGCACCGC   300
GTGGGAGTGG CAGGGCGCTC CGGACAACGT CAAGAGCTAC CCCTATGTTG GCAAGCAGAT   360
CCAGCGCGGC CGCAAGATCA GCGACATCAA CAGCATGCGC ACGTCGGTGT CGTGGACGTA   420
CGATCGGACC GACATCCGTG CCAATGTCGC TTATGATGTC TTCACGGCTC GTGATCCGGA   480
CCATCCCAAC TGGGGCGGCG ACTACGAGCT CATGATCTGG CTCGCCCGCT ATGGCGGCAT   540
CTACCCCATC GGCACGTTCC ACAGCCAGGT CAACCTTGCT GGTCGTACCT GGGATCTCTG   600
GACTGGCTAC AACGGCAACA TGCGTGTCTA CAGCTTCCTC CCCCCGTCCG GCGACATTCG   660
TGACTTCAGC TGCGACATCA AGGACTTCTT CAACTACCTT GAGCGCAACC ATGGCTACCC   720
GGCACGGGAG CAGAATCTGA TCGTCTACCA AGTTGGAACC GAGTGCTTCA CGGGCGGTCC   780
GGCCAGGTTC ACGTGCAGGG ACTTCAGGGC TGACCTGTGG TAAGAGGGGT CATTGGAGTA   840
GGGTGTACTT GCCAGGAAGC GGAGAGGAAC AAGGTAGATA TAATGACAGA CAAGTCATTG   900
GATGCAGTAA ACACGCTTCG TCCTTCATGG GCAAGACTTG CAAGTGCCTA GACAACAGTG   960
CTCAGGGGAC ACAGGGACGA AGATGCATCC TCCAAAACTC AGAAATCGGT AGCAATCGCC  1020
TTTAGAG                                                            1027
```

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 872 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: *Humicola insolens*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
AAAGCCTGAA CACTATTACC ATGTTGCACA GTGTCCTTGC CGGTCTCTTC GCGACTGGAG    60
CGCTCGCCCA GGGCGTGCAT GGCAGCAGTG TGGTGGCGTT GGCTTCTCGG GCTCTACGTC   120
```

CTGTGTGTCC GGTTACACGT GCGTGTACTT GAACGACTGG TACAGCCAAT GCCAGCGCAG 180

CCGACGACGT TACGGACAAC ACAACAACGC CAGGGCAACA TCGACAACAA GGTCAGCCCC 240

GGCTGCCACT TCAACCACTC CGGCCAAGGC GAAGTTCAAG TGGTTGGCAT CAACCAGTCC 300

TGCGCTGAGT TCGGCAAGGA GAGTATCCGG CTATGGGCAA GCACTTACTT CCTTCGCGAC 360

GTCGTCGATT CAAGCGCACA TCAATCGTGG CTTCACATGT CCNNNNNGGC AACTCTGGAC 420

GGCTGACGTG AACGTGACCA ACAACAACTT GGCCGTAGCG ACCGAGAACA AGCTGTGTAC 480

CAGATGCATC AGTACCTCGA CTCGGACGGT CCGGCACGAG CACGGCCTGC GTCAGCACCC 540

AGGTCGGCCT TCAGCGCGTC ATTGGCGCGA CCAACTGGCT CAGGCAAAAC GGCAAGGTTG 600

GACTGCTCGC GACTTGCCGC GGCGCCAAGT GGGTTTGCCA GCAGGCCATT GAGGGCATGC 660

TGAACCACCT CCAGGAGAAT AGCGATGTCT GGACAGGTGC GCTCTGGTGG GCGGGAGGCC 720

CGTGGTGGGG TGACTATATC TAGTCGTTTG AACCTCTTCG GGTATTGGCT ACACCTACTA 780

CAATTCCCTT CTCAAGAAAT ACGTGCCATA GGTGCTATAA GACCGTGGTC CTGACTCAAG 840

AGGGTTTGAC AGGAACGCAG CCCTGAGGCT TT 872

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 368 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Humicola insolens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GTTGAAGGCC CTGAACAGAG GACCTCACGT CCCGAAAATG TCCAGGGCTA CGGAGTATGT 60

ACAGAATATC CACAACCAAA CAAAAGTCAA TTCACCTCCG ATCCCAACAT CACAACCCCT 120

TCAGGCTGGG TCGGAACCAA CTACCTAGCC AGCCTCTCTC CATTTTCCAT CTCCTAACAC 180

CAACCCCCCT CCAATCTCTG CCCCTTAAAT CGGGTTGACC CGAATCGATC CAGATCCGCA 240

AACACCACCT GCCAACCATC CGCTGCGCCC GCCCTGGGTG AAGAACGACT TGGCCGGTCT 300

CGTTGCCGGA GCAGCCACTC GACCAGTAGC TGGCCGCCGT GCGAACTTGC CGTTGTAGTA 360

CCATAGGT 368

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 720 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Humicola insolens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

```
NAAAGGCACC AAGGTGACGG CGTCACCTCG GGCGAGTGGG AGACGATCCG CATCACCGAG     60
AACCACTGGT CGCACGGCCC CGTGACGGAC GTGACCTCGC CGGCCATGAC GTGCTACGAG    120
AAGACGCCCG GCCAGCGGGC CCNAAGACGG TCAACGTGCG GGCCGGCGCG ACCGTCACCT    180
TCACCGTCTA CACGGACGTG GGCCACCCGG GCGCTGCACT TCTACCTGGC CAAGGTGCCG    240
CGGCAAGACG GCCGCGACGT TTGACGGCAA GGGCGCCGTG TGGTTCAAGA TTTACCAGGA    300
CGGCCTGGTG GTTGGGACAG CTCGTTGACC TGGCCATGCT TGGCAAGAAG AGTCTCGTCA    360
ATNNNNNNAC CTGGCCTACG TTGGCAAGAA GAAGTCTCGT CAATCCCCCG TGCGTCCAGG    420
ACGGCGAGTA CCCTGCCGCG TCGAGCACAT TGCCTGCACA GGCGCGAGCG TCGGCGGTGC    480
GCAGCTCTAC ATTTCGTGCG CGCAAATCAA CGTCACGGCG GCACCGGCAC GCTCAACCCG    540
GGCCAGCTCG TCTCGTTCCC GGGCGCCTAC AAGCCCACCG ACCCGGGCAT CCTGTTCCAG    600
CTCTACTGGC CGCCGCCGAC GCAGTACATC AACCCCGGTC CGGCGCCGGT GAAGTGCTGA    660
GTTTGAGTTC ATGAGTACTC CAATGAAGGT TGCGCGGCGG CGAGGGTAGG TCGATAGTTT    720
```

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 724 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Humicola insolens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CCTAGGTCGC CCACCATGCG CGTTTCTCTT GCTCTCCTCG CCTACCTGCT CAGCGCCGCC 60

CCGGCCTCGC CCGTCCCGGA GCTCGAGCCC CGGCAGTCCG GCAACCCCTT CTCCGGCCGC 120

ACCCTGCTGG TCAACTCGGA CTATAGCAGC AAGCTCGACC AGACGCGCCA GGCCTTTCCT 180

GTCCCGCGGC GACCAGACCA ACGCTGCCAA GGTCAAGTAC GTCCAGGAGA AGGTTGGCAC 240

CTTTCTATTG GACTTCCAAC ATCTTCCTCC TGCGCAGCAC TGACGTTGCC ATCCAGAATG 300

CGCGCCGCCA AGGCCGCGCG AGAACCCCAT CGTCGGTCTC GTCCTGTACA ACCTCCCCGA 360

CCGCGACTGC AGCGACGCGG CAGTACCTCT GGCGACGTTA AGCTCTCCCA GAACGGCCTG 420

AACCGGTACA AGAACGAGTA CGTCAACCCG TTCGCCCAGA AGCTCAAGGC CGCGTCCGAC 480

GTGCAGTTCG CCGTCATCCT CGAGCCCGAT GCCATCGGCA ACATGGTCAC GGGCACCAGC 540

GCCTTCTGCC GCAACGCCCG CGGCCCTCAG AGGAGGCCAT CGGCTATGCT ATCTCTCCTC 600

GGCTGGGCCG ATAAGCTCGA GCCAACTGCC CAGGAGGTGC CACCATCCTC CAAAAGGCCG 660

GTAACAACGC AAGATCGCGG CTTCTCAGCA ACGTTCCAAC TACAACCTAT TCACGACAAC 720

CGCG 724

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Humicola insolens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CCTCCTTCCA GTCTTTGAGT TCCTTCGGAC CTGCAGCGTC CTGAACAACT CGCTCTAGCT 60

CAACAACCAT G 71

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 572 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Humicola insolens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

```
GGCCGTCGCC GCTTCTTCAG TTGTGTACGA TCATCCAGCA ACTCGCAGTT CACCATGGTC    60
TCGCTCAAGT CTGTCCTCGC GGCCGCCACG GCTGTGAGCT CTGCCATTGC TGCCCCTTTT   120
GACTTCGTTC CTCGGGACAA CTCGACGGCC CTTCAGGCTC GCCAGGTGAC CCCCAACGCC   180
GAGGGCTGGC ACAACGGCTA CTTCTACTCG TGGTGGTCCG ACGGCGGAGG CCAGGTTCAG   240
TACACCAACC TCGAGGGCAG CCGCTACCAG GTCAGATGGC GTAACACCGG CAACTTCGTC   300
GGTGGTAAGG GTTGGAACCC GGGAACCGGC CGCACGATCA ACTACGGCGG CTACTTCAAC   360
CCCCAGGGCA ACGGCTACCT GGCCGTCTAC GGCTGGACCC GCAACCCGCT CGTCGAGTAC   420
TATGTCATCG AGTCGTACGG CACGTACAAT CCCGGCAGCC AGGCTCAGTA CAAGGGCACA   480
TTCTATACCG ACGGCGATCA GTATGACATC TTTGTGAGCA CCCGTCACAA CCAGCCCAGC   540
ATCACGGCAC CCGGACGTCC AGCTAGTACT GG                                 572
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(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 173 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Humicola insolens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

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AAAGTAGATC GTCCTGCTCC CTAGAAACCA GTCACTCATT CACAATGCGT TCTATCGCTC    60
TCGCTCTTGC TGCGGCTCCG GGCCTCCTCG CCCAGTCCCA GCTCTGGGCC AGTGCGGTGG   120
CATCGGCTGG AACGGCCTAC GACTTGCGTC TCGGGCGCTA CCTGCACCAA GAT          173
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(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 214 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Humicola insolens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

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AAAGCCGCAG CGATCAAGAA CCCAAGCAGT CTGTCAAAAT GGTCGCCTTC TCGTCCCTCT    60
TCCTCGGTGC TTCCATCGCC GCCACGCGTC GGCCGGCCCC GGTGTAGCTG CCCGGCATGC   120
ACCTGAACAA GCGTCAGACC TACACCCCAG AGCGCTACCG GCACTCACAA CGGCTACATC   180
TTCTCCTTCT GGACTGACGG TCTGGCCGAA CGTC                                214
```

CLAIMS

1. A method of screening for a DNA sequence coding for a protein of interest, the method comprising

(a) cloning, in suitable vectors, a DNA library from an organism suspected of producing one or more proteins of interest,

(b) transforming suitable yeast host cells with said vectors,

(c) culturing the host cells under suitable conditions to express any protein of interest encoded by a clone in the DNA library, and

(d) screening for positive clones by determining any activity of a protein expressed in step (c).

2. A method according to claim 1, wherein the DNA library is a cDNA library prepared from the mRNA of an organism suspected of producing one or more proteins of interest.

3. A method according to claim 1 or 2, wherein positive clones isolated in step (d) are subjected to rescreening, reisolation and recloning.

4. A method according to claim 1 or 2, wherein the organism suspected of producing one or more proteins of interest is a eukaryotic organism.

5. A method according to claim 4, wherein the eukaryotic organism is a fungus.

6. A method according to claim 4, wherein the eukaryotic organism is a plant.

7. A method according to any of claims 1-6, wherein the protein of interest is an enzyme.

8. A method according to claim 1, wherein the yeast host cell is a strain of Saccharomyces cerevisiae, Schizosaccharomyces pombe, Hansenula, Pichia, Yarrowia lipolytica or Kluyveromyces lactis.

9. A process for producing a protein of interest in a heterologous host cell, the process comprising transforming a suitable heterologous host cell with a DNA sequence coding for a protein of interest, which DNA sequence has been isolated by the method of claim 1, culturing the transformed cells under suitable conditions to express the protein, and recovering the expressed protein from the culture.

10. A process according to claim 9, wherein the DNA sequence coding for the protein of interest has been isolated by the method of claim 3.

11. A process according to claim 9 or 10, wherein the protein of interest is an enzyme.

12. A process according to any of claims 9-11, wherein the host cell is a strain of Aspergillus, for instance a strain of Aspergillus oryzae or Aspergillus niger.

13. An enzyme which exhibits cellulase activity, and which has the following characteristics

(a) the DNA sequence encoding the enzyme has been isolated from a DNA library of Humicola insolens,

(b) said DNA sequence comprises at least one of the following partial sequences

(i) TGGCAGCAGT GTGGTGGCGT TGGCTTCTCG GGCTCTACGT CCTGTGTGTC CGGTTACACG TGCGTGTACT TGAACGACTG GTACAGCCAA TGC
(SEQ ID#1)

(ii) CAGCGCAGCC GACGACGTTA CGGACAACAC AACAACGACC AGGGCAACAT CGACAACAAG GTCAGCCCCG GCTGCCACTT CAACCACTCCG G
(SEQ ID#2)

(iii) CCAAGGCGAA GTTCAAGTGG TTGGCATCAA CCAGTCCTGC GCTGAGTTCG GCAAGGAGAG TATCCGGCTA TGGGCAAGCA CTTACTTCCT TCGCGACGTC GTCGATTCAA GCGCACATCA ATCGTGGCTT CA (SEQ ID#3)

(iv) CTGACGTGAA CGTGACCAAC AACAACTTGG CCGTAGCGAC CGAGAACAAG CTGTGTACCA GATGCATCA (SEQ ID#4)

(v) GGACGGTCCG GCACGAGCAC GGCCTGCGTC AGCACCCAGG TCGGCCTTCA GCGCGTCATT GGCGCGACCA ACTGGCTCAG GCAAAACGGC AAGGTTGGAC TGCTCGCGAC TTGCCGCGGC (SEQ ID#5)

(vi) GCCAAGTGGG TTTGCCAGCA GGCCATTGAG GGCATGCTGA ACCACCTCCA GGAGAATAGC GATGTCTGGA CAGGTGCGCT CTGGTGGGCG GGAGGCCCGT GGTGGGGTTG ACTATATCTA (SEQ ID#6)

(c) the enzyme comprises a cellulose-binding domain, and

(d) the enzyme exhibits endocellulase activity in the presence of linear alkyl benzene sulfonate.

14. An enzyme according to claim 13, a crude extract (15 µl) of which diluted with one volume of 0.15% linear alkyl benzene sulfonate and added to a 2% agarose gel containing 1% carboxymethyl cellulose in 50 mM sodium phosphate buffer,

pH 7, mixed with one volume of 0.15% linear alkyl sulfonate forms a clearing zone in said agarose gel after 18 hours of incubation, which clearing zone is equal to (less 3 mm) the clearing zone formed in a similar carboxymethyl cellulose gel not containing any linear alkyl benzene sulfonate, provided that the concentration of enzyme in the extract is such that a clearing zone of at least 10 mm is formed in a carboxymethyl cellulose gel (with no linear alkyl benzene sulfonate) under the conditions specified above.

15. A detergent additive comprising an enzyme exhibiting cellulase activity according to any of claims 13-14, preferably in the form of a non-dusting granulate, stabilised liquid or protected enzyme.

16. A detergent additive according to claim 15, which further comprises one or more other enzymes such as a protease, amylase, lipase or peroxidase.

17. A detergent composition comprising an enzyme exhibiting cellulase activity according to any of claims 13-14.

18. A detergent composition according to claim 17, which further comprises a linear alkyl benzene sulfonate surfactant.

19. A detergent composition according to claim 17 or 18, which further comprises one or more other enzymes such as a protease, amylase, lipase or peroxidase.

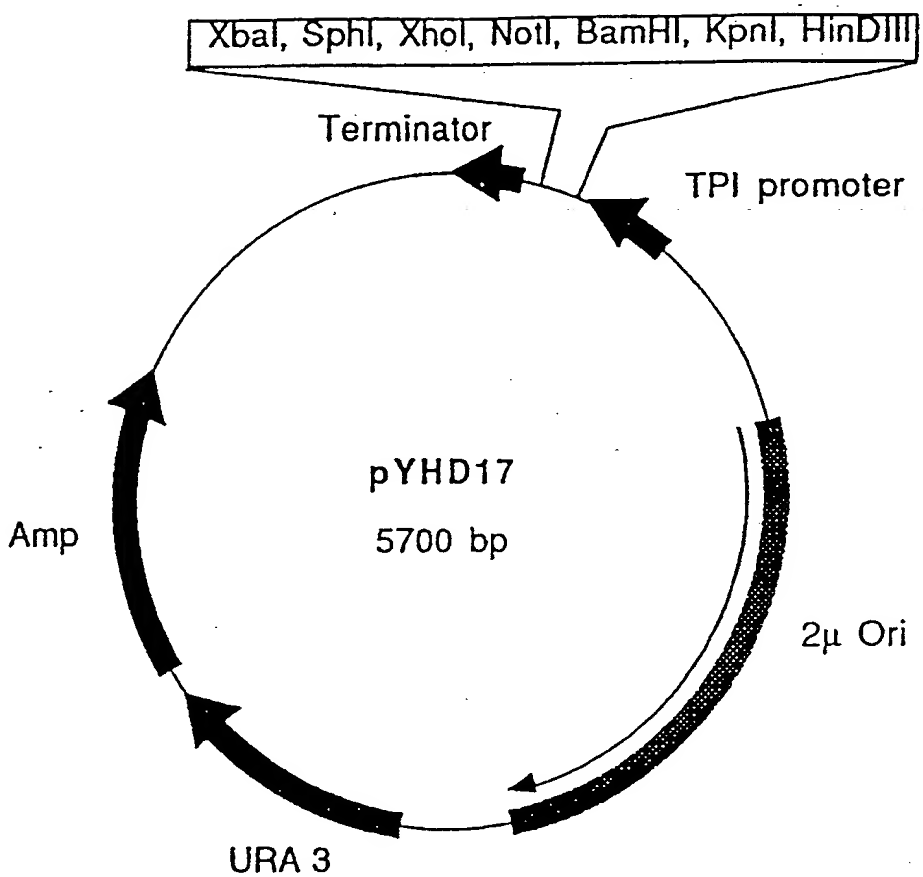
XbaI, SphI, XhoI, NotI, BamHI, KpnI, HinDIII
Terminator
TPI promoter
pYHD17
5700 bp
Amp
2µ Ori
URA 3

Fig. 1

2/2

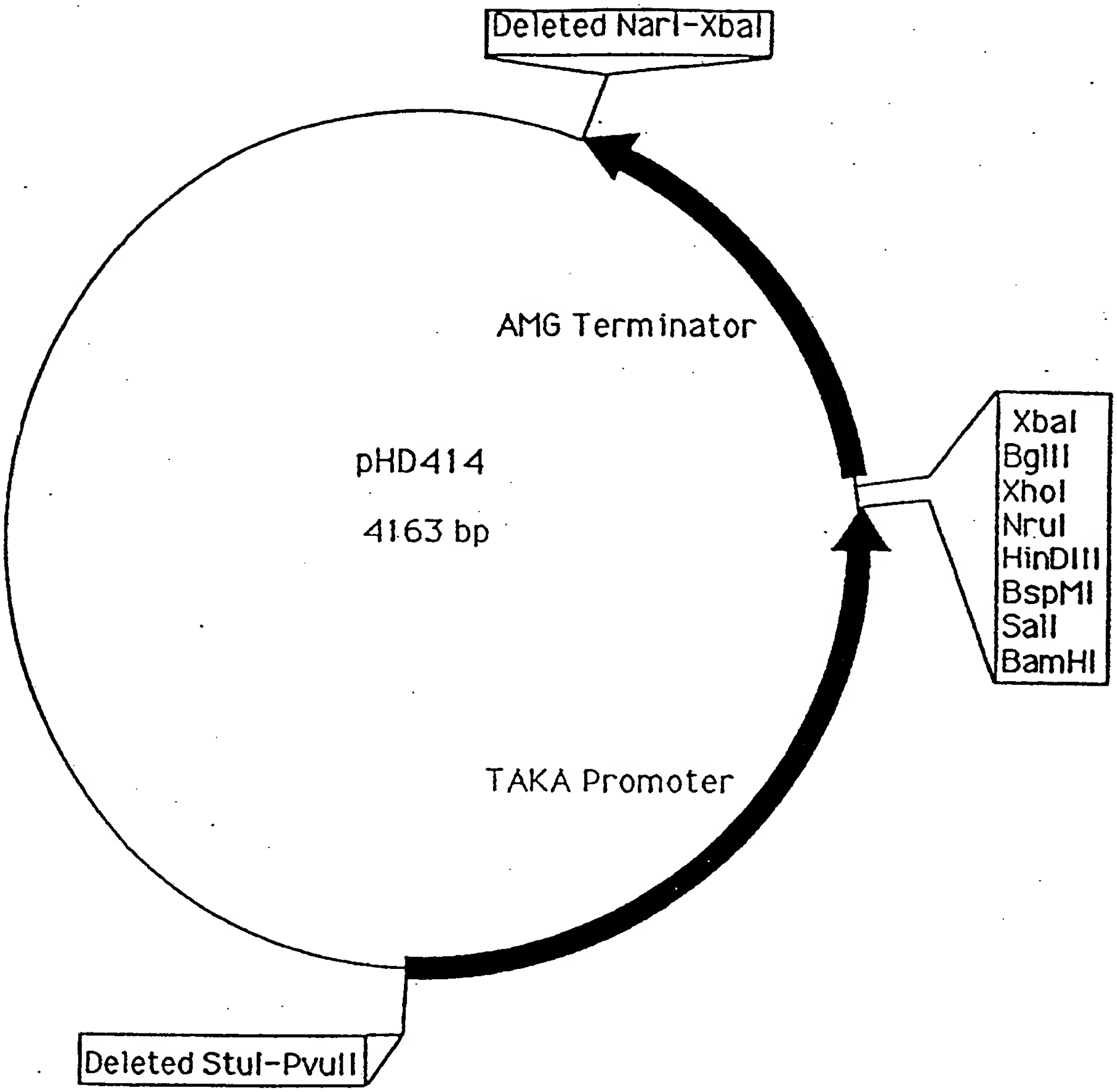
Deleted NarI-XbaI
AMG Terminator
pHD414
4163 bp
XbaI
BglII
XhoI
NruI
HinDIII
BspMI
SalI
BamHI
TAKA Promoter
Deleted StuI-PvuII

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 92/00360

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C12N 15/81, C12N 1/16, C12N 9/42
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C12N, C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO, A1, 9201069 (TRANSKARYOTIC THERAPIES, INC.), 23 January 1992 (23.01.92), page 9, line 14 - line 30, claims 1-2	1-12
	--	
X	PROC.NATL.SCI., Volume 80, July 1983, Gary L. McKnight et al, "Selection of functional cDNAs by complementation in yeast" page 4412 - page 4416	1-12
	--	
X	SCIENCE, Volume 236, 1987, David T. Burke et al, "Cloning of Large Segments of Exogenous DNA into Yeast by Means of Artificial Chromosome Vectors", page 806 - page 812, page 808 right column line 10-12	1-12
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" erlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 April 1993	20 -04- 1993
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 92/00360

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chemical Abstracts, Volume 114, No 5, 4 February 1991 (04.02.91), (Columbus, Ohio, USA), Okazaki, Koei et al, "High-frequency transformation method and library transducing vectors for cloning mammalian cDNAs by transcomplementation of Schizosaccharomyces pombe", page 202, THE ABSTRACT No 37151y, Nucleic Acids Res. 1990, 18 (22), 6485-6489 --	1-12
A	THE EMBO JOURNAL, Volume 8, No 12, 1989, David P. Gearing et al, "Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor" page 3667 - page 3676 --	1-12
A	WO, A1, 8909259 (NOVO INDUSTRI A/S), 5 October 1989 (05.10.89), see claim 9 and page 3, page 7 and example 1 --	13-19
A	WO, A1, 9117243 (NOVO NORDISK A/S), 14 November 1991 (14.11.91) --	13-19
A	US, A, 4435307 (NOVO INDUSTRI A/S), 6 March 1984 (06.03.84) --	13-19
A	Chemical Abstracts, Volume 110, No 19, 8 May 1989 (08.05.89), (Columbus, Ohio, USA), Rao U. Subrahmanyeswara et al., "Purification and characterization of a beta-glucosidase and endocellulase from Humicola insolens", page 358, THE ABSTRACT No 168875c, Indian J. Biochem. Biophys. 1988, 25 (6), 687-694, (e) --	13-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 92/00360

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Chemical Abstracts, Volume 71, No 21, 24 November 1969 (24.11.69), (Columbus, Ohio, USA), Ramabadran R. et al., "Cellulase of Humicola insolens", page 33, THE ABSTRACT No 98499h, Indian J. Exp. Biol. 1969, 7 (3), 186-187, (e)	13-19
	--	
A	Chemical Abstracts, Volume 109, No 23, 5 December 1988 (05.12.88), (Columbus, Ohio, USA), Hayashida Shinsaku et al., "Cellulases of Humicola insolens and Humicola grisea", page 295, THE ABSTRACT No 207112c, Methods Enzymol. 1988, 160, 323-332, (e)	13-19
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 92/00360

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

The inventions claimed are composed of the following two different inventions:

I. Claims 1-12 directed to a method of screening for a DNA sequence coding for a protein of interest and a process for producing the protein.

II. Claims 13-19 directed to an enzyme and its use. The "special technical features" of group I relate to a method of producing a protein in yeast by recombinant DNA-technique while the "special technical features" of group II relate to an enzyme from Humicola insolens which exhibits cellulase activity. Thes groups of inventions are not so linked as to form a single general inventive concept. There is no technical relationship among those inventions involving one or more of the same or corresponding technical features.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 92/00360

Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)

Method of screening for a DNA sequence coding for a protein of interest, the method comprising

(a) cloning, in suitable vectors, a DNA library from an organism suspected of producing one or more proteins of interest,

(b) transforming suitable yeast host cells with said vectors,

(c) culturing the host cells under suitable conditions to express any protein of interest encoded by a clone in the DNA library, and

(d) screening for positive clones by determining any activity of a protein expressed in step (c).

An enzyme which exhibits cellulase activity and has been isolated from DNA library of Humicola insolens. The enzyme has a cellulosebinding domain and exhibits endocellulase activity in the presence of linear alkyl benzene sulfonate.

INTERNATIONAL SEARCH REPORT

Information on patent family members

26/02/93

International application No.
PCT/DK 92/00360

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9201069	23/01/92	AU-A- 8299191	04/02/92
WO-A1- 8909259	05/10/89	EP-A- 0406314	09/01/91
		JP-T- 3504080	12/09/91
WO-A1- 9117243	14/11/91	AU-A- 7887491	27/11/91
US-A- 4435307	06/03/84	AT-B- 384442	10/11/87
		AT-B- 394574	11/05/92
		BE-A- 888632	29/10/81
		CH-A- 663511	31/12/87
		DE-A,C- 3117250	01/04/82
		FR-A,B- 2481712	06/11/81
		GB-A,B- 2075028	11/11/81
		JP-C- 1443072	08/06/88
		JP-A- 57023699	06/02/82
		JP-B- 61016316	30/04/86
		NL-A- 8102123	16/11/81